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Molecular Dissection of the Mechanism of ssDNA-binding Proteins

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Kelly Binns is a senior majoring in Chemistry with a concentration in Biochemistry.
This project was funded by a 2013 Adrian Tinsley Summer Research Grant mentored by Dr. Boriana Marintcheva (Biology). Kelly presented her research at the 2014 National Conference on Undergraduate Research (NCUR) in Lexington, KY. Kelly plans to pursue a career in biomedical research after studying molecular and cell biology in graduate school.

The maintenance of the integrity of DNA is crucial for life as any change could result in cellular abnormalities leading to disease. DNA is most stable as a double helix. However, many processes manipulating DNA require the presence of vulnerable single-stranded DNA (ssDNA). ssDNA-binding proteins (SSBs) have the ability to bind to ssDNA, stabilize it and thus allow DNA transactions to take place. Prokaryotic SSBs, found in bacteria and viruses that infect them, are comprised of a DNA-binding body and a negatively-charged flexible C-terminal tail. The removal of the tail results in increased ability of the protein to bind ssDNA. The goal of this project is to dissect the roles of the flexibility and negative charge of the tail for biological function using gp2.5, the ssDNA-binding protein of bacteriophage T7, as a prototype for prokaryotic SSB.

The natural form of gp2.5 and a mutant lacking the C-terminal tail have been successfully expressed in *BL21 (DE3) E.coli* cells and purified using His-tagged technology. The generation of a mutant with an uncharged tail is currently in progress. Once this mutant protein is produced, the ssDNA binding abilities of all three versions of gp2.5 will be evaluated in order to better understand how SSBs and other proteins with flexible charged tails work. Overall, this study has the potential to contribute to selection of antibacterial agents that kill bacteria by disrupting the function of their ssDNA-binding proteins.

Introduction

Single-stranded DNA-binding proteins (SSBs) are keystones in the processes involving DNA, the genetic blueprint of all living organisms. Maintaining the integrity of the DNA code is crucial to life. Alterations in DNA can result in malfunctioning proteins which lead to cellular abnormalities, potentially instigating debilitating diseases.

DNA is most stable in its double-stranded form, commonly described as a double helix. The double helix is formed from two single-stranded DNA (ssDNA) chains that are held together by hydrogen bonds between complementary bases. However, many of the processes, which manipulate this critical molecule, require the two strands to be separated in order for the genetic material to be copied or processed. In this single stranded form, the DNA is highly prone to the attack of nucleases, enzymes that will cut and essentially

destroy the genetic material (Shereda et al., 2008). There is also a high potential for the two strands to rejoin before any operation with DNA can be completed. SSBs provide a solution for both problems by binding to and stabilizing the single-stranded conformation, while also shielding ssDNA from nucleases (Shereda et al., 2008). They also facilitate replication, recombination and repair by removing DNA secondary structures that impede the progress of all enzymes involved in these DNA transactions (Shamoo, 2002). Another critical role of SSBs is recruitment of specialized proteins involved in DNA manipulations in cells (Shereda et al., 2008). Due to this critical role in the maintenance of DNA, SSBs are present in all cells and are essential to life. In addition, viruses code for their own ssDNA-binding proteins despite the fact that their host already expresses these critical proteins (Borjac-Natour et al., 2004). The way in which SSBs work is elucidated by their structure.

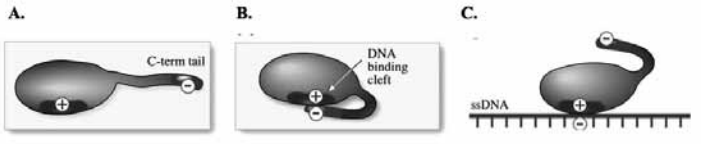
All known ssDNA-binding proteins share a similar 3D-structure referred to as the oligosaccharide/oligonucleotide-binding fold or OB fold domain (Shamoo, 2002), as depicted in Figure 1. A protein domain is a specific structural component that is responsible for one of the important tasks in a protein's overall function. Domains are analogous to the parts of a car. For instance, steering wheels are specific structural features that are nearly identical in every type of car and perform the identical function of allowing a driver to direct the car's movement. The contributing role of the OB fold in SSBs is to facilitate the recognition and binding of single-stranded DNA (Shamoo, 2002). The name, Oligosaccharide/oligonucleotide-binding fold, was termed for the part of DNA to which it binds. Oligosaccharides or sugars make up part of the negatively-charged backbone of DNA. The OB fold, consists of five anti-parallel Beta sheets constituting the shape of a barrel and capped by an alpha helix, contains a narrow cleft that is able to distinguish single-stranded DNA from the more expansive double stranded DNA (Shamoo, 2002). The positively-charged walls of the DNA-binding cleft interact with the negatively charged DNA backbone, while the aromatic residues of the cleft form stacking interactions with the nucleotide bases, ensuring the secure binding of ssDNA to the OB fold (Shamoo, 2002).

Figure 1. Three-dimensional Structure of Gp2.5, ssDNA-binding Protein of Bacteriophage T7.

Gp2.5 is a typical prokaryotic ssDNA-binding protein containing an OB fold and flexible negatively charged tail. The OB fold contains the ssDNA binding cleft of the protein marked by a bracket. The C-terminal tail is depicted by the chain of letters at the end of the protein. These letters represent the sequence of amino acids building the tail. The letters "E", "D" and "F" represent the amino acids glutamate, aspartate and phenylalanine that contribute to the negative charge of the tail. The determination of the structure of T7 gp2.5 is described by Hollis *et al.,* 2001. The above figure appeared originally in Marintcheva *et al.,* 2008

In addition, all prokaryotic type ssDNA-binding proteins have an acidic C-terminal tail which mediates protein-protein interactions with other proteins involved in the replication of DNA (Shereda et al. 2008). Furthermore, studies have suggested that the tail is critical for the protein to maintain a stabilized dimer configuration (Hollis et al., 2001). The C-terminal tail can be envisioned as a tail that hangs off of the end of the protein, terminating in a carboxyl group (Figure 2). This C-terminal tail is also very flexible and negatively charged. In the absence of DNA, the C-terminal tail interacts with the positively-charged fissure of the OB fold, protecting the DNA-binding cleft from randomly sticking to negatively-charged molecules (Figure 2). As a result of this interaction, the C-terminal tail shields/competes with DNA for this site (Marintcheva et al., 2008). The effect of this competition has been extensively studied on the model of bacteriophage T7 ssDNA-binding protein, which is considered a prototype for prokaryotic ssDNA-binding protein.

Figure 2. Proposed Mechanism of Action of Prokaryotic ssDNA-binding Proteins.



It has been proposed that in the absence of DNA (Panel A and B) the C-terminal charged tail of SSB fluctuates between bound and free state. In the bound state (panel A), it occupies the DNA binding cleft, whereas in the free state, the tail extends away from the protein (panel B). In the presence of DNA, the tail is displaced from the binding cleft and is continuously available for interactions with other proteins involved in DNA transactions. The area marked with a plus sign, represents the DNA-binding cleft. The area of the C-terminal tail marked with a minus sign depicts the portion of the tail that is negatively charged. The above model was originally proposed by Kowalczykowski *et al.,* 1981.The figure panels were originally published in Marintcheva *et al.*, 2008.

T7 is a bacterial virus that infects *Escherichia coli*, a common inhabitant of our gastrointestinal tract. Bacteriophage literally means bacteria eater, a definition that fits well with the fast pace of bacteriophage replication and cellular destruction. For example, once T7 infects *E.coli* it takes less than an hour for the cell to be destroyed and hundreds of new bacteriophages to be released to the extracellular environment. T7 is a well established model system to study the mechanism of replication due to the involvement of a minimal number of players and its rapid growth. Thus, T7 allows straightforward analysis and identification of basic principles that are applicable to the living world in general. For example, the basic principles of replication are the same in T7 and in human cells. However, T7 duplicates its DNA with only five players, whereas human cells accomplish the same job with a double digit number of players. Yet, in its essence, the process works the same way and what is learned about T7 guides scientists in what to look for in humans. In addition, studies of T7 replication have brought to light many molecular biology tools, including the enzyme used to sequence the human genome.

The T7 ssDNA binding protein is referred to as gene 2.5 protein or gp2.5 according to its position along T7 genome. The C-terminal tail of this SSB has been extensively mutated to identify key determinants of its function. Although the removal of the tail had detrimental effects on replication *in vivo*, this deletion increases the binding of the protein to ssDNA, presumably because it removes the competitive interaction between the DNA and the C-terminal tail. In addition, it has been demonstrated that the gradual removal of the tail also results in a gradual increase in the ability of the gp2.5 to bind to ssDNA (Marintcheva et al., 2006). Although the effects of removing and shortening the tail are well known, the performed experiments do not distinguish between the contribution of charge and the flexibility of the tail. Genetic data suggests that charge is important for function since a mutant in which negatively charged amino acids, the building blocks of proteins, are converted to non-charged ones fails to support viral growth (Marintcheva et.al, 2008). However, the hypothesis has not been directly tested biochemically. The goal of this project was to test the hypothesis that negative charge is essential for the function of the C-terminal tail of gp2.5. We aimed to express and purify the **W**ild **T**ype or the natural form of the protein, gp2.5-**WT**, a mutant with **N**o **T**ail (gp2.5-**NT**) and a mutant with the full length tail but **N**o **C**harge (gp2.5-**NC**) so that we can then compare the ssDNA-binding abilities of all three forms.

This study is expected to contribute to the general understanding of how flexible charged tails function in proteins that are associated with DNA transactions. This in turn may contribute to studies focused on using these proteins as potential drug targets. A recent study has revealed the potential benefit of utilizing SSBs as antibacterial targets. Results showed that all three of the tested compounds, designed to disrupt SSB activity, led to cell death for several evolutionarily diverse bacterial species (Marceau et al., 2013). This application holds the potential benefit of eliminating the barrier of prevailing antibiotic resistant strains of bacteria. Another potential application involves histone proteins that package DNA in human cells. These proteins have a flexible charged tail similar to that of gp2.5. Chemical modifications changing the charge of the histone tails regulate gene expression and are considered potential targets for cancer therapy (Činčárová et al., 2012). This project has the potential to contribute to these studies seeking to develop therapeutic agents, targeting SSBs or other similar proteins with flexible charged tails.

Materials and Methods

Plasmids: The following plasmids were used in this study: The plasmid pET-17.b-gp2.5-NC, encoding mutant gp2.5 with full size tail but no charge (Marintcheva et al., 2006), pET-19.b-PPS-gp2.5 WT, encoding the His-tagged version of the natural form of gp2.5; pET-19.b-PPS-gp2.5-Δ26C, coding for the His-tagged version of gp2.5 lacking the C-terminal tail (Hollis et al., 2002). For the purpose of our study we are naming this version of gp2.5 "gp2.5-No Tail" or gp2.5-NT for short. All plasmids were a gift from Dr. Charles C. Richardson (Harvard Medical School).

***E coli* Strains:** The *DH5α* strain was used to propagate all plasmids and *BL21 (DE3)* strain was used for protein expression.

Transformation: Transformation for the purpose of plasmid propagation and protein expression was conducted by pipetting 50µl of competent cells into a 1.5 ml eppendorf tube with 1µl plasmid of interest, followed by 10 minutes of incubation on ice. Following heat-shock at 42°C for 40 seconds, the reactions were cooled on ice and supplemented with 500µl of Luria Broth (LB) media. The cells were grown for 1 hr while shaking at 37°C and plated on LB/ampicillin to grow overnight at 37°C.

Plasmid Preparation: Single colonies of cells were incubated overnight in LB supplemented with 100µg/µl ampicllin at 37°C. Plasmid DNA was purified using the QIAGEN plasmid purification kit as recommended by the manufacturer.

Cloning of gp2.5-NT: Cloning of Gp2.5-NC into a pET19.b PPS vector was attempted, so that the protein could be purified using His-tagged technology. Vectors, pET 19.b PPS-

gp2.5-NT, and17.b-gp2.5-NC were digested with restriction enzymes, BamHI-HF and NdeI. Digestion reactions were run on a 0.8% agarose/1X TBE gel at 100 volts for verification. The desired fragments were extracted from the gel and purified using QIAquick Gel Extraction kit as recommended by the manufacturer. Fragments were ligated using the New England BioLabs Quick Ligation kit in accordance with the manufacturer's recommendations. Ligation reactions were transformed into *DH5*α cells and single colonies selected. The subsequently isolated plasmids were sequenced at Eurofins MWG Operon.

Protein Expression: Single transformants were inoculated in LB media/ampicillin supplemented with 1% glucose and grown overnight. 10 ml of overnight cultures were added to 300 ml of fresh LB/ampicillin (100µg/ml) and were grown to an optical density of 0.7 at 600 nM. Protein expression was induced with 1mM final concentration of IPTG (isopropyl-1-thio-β-D-galactopyranoside) for 3 hours. Cells were collected via centrifugation at 4°C and 5000rpm for 10 minutes. Pellets were resuspended in 5 ml of Buffer B containing 70mM Imidazole, 500mM Tris-Cl and 500mM NaCl, as previously described in Rezende *et al.*, 2002. All buffers used for purification contained 1mM (final concentration) AEBSF, 1X cOmplete EDTA-free protease inhibitor cocktail tablets, and 1mM DTT.

Protein Purification: The protein purification procedure was performed as previously described in Rezende *et al.,* 2002. In brief, cells were broken open with addition of 1mg/ml lysozyme and were rocked at 4°C for 2 hours. Benzonase (6.25units/ml) was added to reduce viscosity and samples were warmed to 20°C in a 37°C water bath. Soluble proteins were separated from cell debris by centrifugation at 8,000 *g* and 4°C for 30 minutes. Supernatant was loaded on a 2ml nickel-NTA agarose column. Non-specific proteins were washed away with 20 ml of Buffer B. Gp2.5 protein was eluted with 2 column volumes of Buffer B supplemented with 500mM imidazole. Gp2.5 protein was dialyzed against Buffer S containing 50mM Tris-Cl, 0.1mM EDTA, 50% glycerol, and 1mM DTT. Buffer S was supplemented with 150mM NaCl for dialysis of gp2.5-NT, and gp2.5-NC. All protein samples were stored at -20°C.

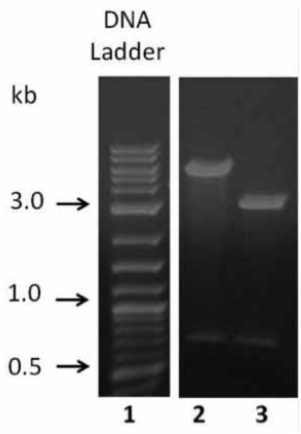
DNA-binding Activity Assay: The activities of gp2.5-WT and gp2.5-NC were assessed using M13 circular ssDNA as a template. The 20µl reactions contained 1µg M13 ssDNA, 15mM $MgCl_2$, 5mM DTT, 50mM KCl, 10% glycerol, and 20µM of either gp2.5-WT or gp2.5-NT. 1X loading dye (final concentration) was added to each sample. Proteins were diluted with buffer containing, 20mM Tris-Cl (pH 7.5), 1mM DTT, and 500µg/ml bovine serum albumin. The samples were run on a 0.8% agarose/1X TBE gel in 0.5% Tris-Glycine buffer. The gel was run on ice at 100 volts.

Results

Cloning of gp2.5-NC into pET 19bPPS

The following plasmids were digested with restriction enzymes, BamHI-HF and NdeI: pET 19.b PPS -gp2.5-NT, and pET17.b-gp2.5-NC. The digested products were run on a 0.8% agarose/1X TBE gel at 100 volts (Figure 3). The gel was stained with ethidium bromide and analyzed under ultraviolet light. The backbone of pET 19.bPPS appeared as a single band at approximately 6kb (lane 1, Figure 3). The fragment of pET 17.b containing the region coding for gp2.5-NC appeared as a single band at approximately 0.7kb (Figure 3, lane 2). Both fragments were extracted, purified, and ligated using the NEB Quick Ligation kit. The ligation reactions were transformed into a *DH5*α strain of *E.coli* cells and single colonies selected. The sequencing results of the plasmids isolated from the selected colonies, obtained from Eurofins MWG Operon, identified all isolated plasmids as uncut 19.b PPS- gp2.5-NT.

Figure 3. Verification of Digestion Products and Subsequent Ligation

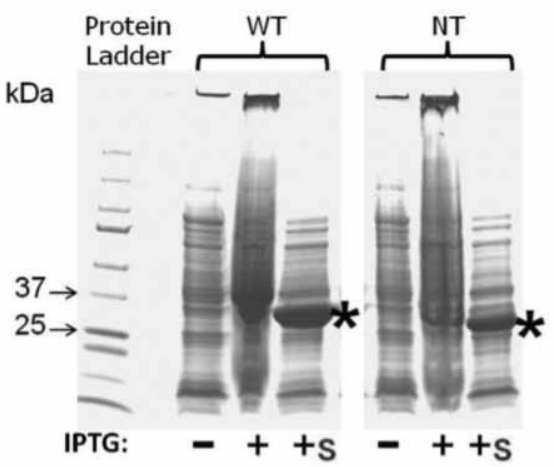


A. Plasmids of interest were digested with NdeI and BamHI restriction enzymes and the reaction products were analyzed on a 0.8% agarose/1X TBE gel at 100 volts. **1** - TriDye 2-Log DNA Ladder; **2** - pET 19.b PPS–gp2.5-NT; **3** - pET17.b-gp2.5- NC.

Protein Expression

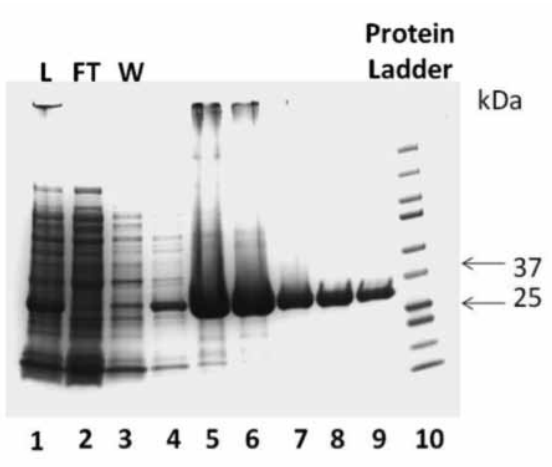
Plasmids coding for gp2.5-WT and gp2.5-NT were transformed in *BL21 (DE3)* competent cells. Single colonies were used to grow an overnight starter culture, which was subsequently diluted into 300ml of LB/ampicillin. Protein expression was induced by the addition of IPTG. Three hours following induction, cells were harvested by centrifugation and the samples were analyzed on a 4-20% mini-PROTEAN TGX/1X Tris Glycine gel at 100 volts (Figure 4), followed by staining of the gel with Bio-Safe Coomassie Blue G-250 . The expected mass of gp2.5-WT and gp2.5-NT, calculated from the amino

Figure 4. Protein Expression.



Protein expression was monitored on a 4-20% mini-PROTEAN TGX/ 1X Tris Glycine gel at 100 volts. Precision Plus Protein Kaleidoscope protein ladder (far left) was used as a reference for molecular weight. Samples of gp2.5-WT (WT) and gp2.5-NT (NT) were loaded as follows (left to right): uninduced (-), induced (+), induced soluble proteins (+s). Bands at ~25 kDa and ~22kDa, respectively are marked with a *.

Figure 5. Gp2.5-NT Purification.



Protein purification fractions were analyzed on a 4-20% mini-PROTEAN TGX/ 1X Tris - Glycine buffer gel at 150 volts. Precision Plus Protein Kaleidoscope was used as a reference for molecular weight. Samples were loaded as follows: **1** – lysate (L), **2** – Flow Through (FT), **3** – Wash (W), **4** – Eluted fraction (EF) 1, **5** -EF 4, **6** – EF 7, **7** – EF 11, **8** – EF 13, **9** – EF 15, **10** - Precision Plus Kaleidoscope Protein ladder.

acid sequences is 25.7 kDa and 22.68 kDa respectively. A dark band between 25 and 37 kDa was observed in the induced (the lanes marked with + as depicted in Figure 4) gp2.5-WT sample and between 20 and 25 kDa in the induced gp2.5-NT sample (as represented by "*" in Figure 4). As expected the proteins of interest were found to be soluble (the lines marked with "$+^{s}$" in Figure 4).

Protein Purification

Purification of gp2.5-WT and gp2.5-NT was evaluated on a 4-20% mini-PROTEAN TGX/ 1X Tris - Glycine buffer gel at 150 volts (Figure 5), followed by staining of the gel with Bio-Safe Coomassie Blue G-250. As expected, several dark bands appeared in lanes loaded with Lysate (L), Flow-Through (FT) and Wash (W) fractions (lanes 1-3). A dark band was resolved between 25 and 37 kDa, along with several other faint bands, indicating the elution of gp2.5 with contaminants in eluted fractions 1, 4 and 7 (lanes 4, 5, and 6, respectively) A single dark band appeared for eluted fractions 11, 13, and 15 (lanes 7, 8, and 9, respectively) between 25 and 37 kDa.

To ensure that pooled and subsequently dialyzed fractions contained pure gp2.5-WT or gp2.5-NT, one microgram of each purified protein was run on a 4-20% mini-PROTEAN TGX/ 1X Tris - Glycine gel (Figure 6-A). Lanes 1 and 2 (gp2.5-WT and gp2.5-NT respectively) showed a single distinct band aligning with the appropriate molecular weight (MW) marker for their expected sizes.

Isolation of Pure and Active gp2.5-WT and gp2.5-NT

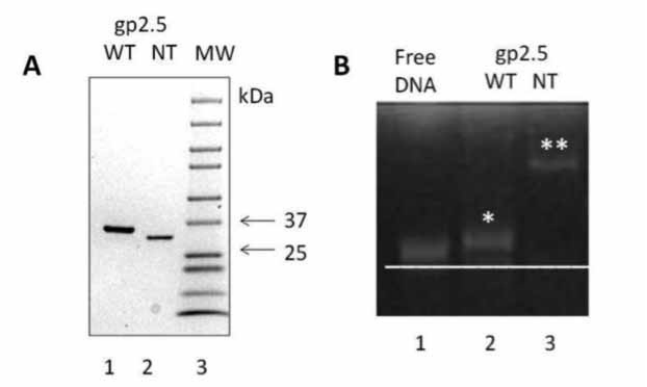
To verify that pure gp2.5-WT and gp2.5-NT was successfully isolated without compromising their activity, purified gp2.5-WT and gp2.5-NT were run on a 0.8% agarose gel/1X TBE buffer gel at 100 volts in the presence of circular single-stranded DNA (Figure 6B). DNA incubated with gp2.5-WT and gp2.5-NT (lanes 2 and 3 respectively (Figure 6B) moved through the agarose gel more slowly than free DNA (lane 1 Figure 6B). Gp2.5-NT movement through the gel was slower than that of gp2.5-WT.

Discussion and Conclusions

Gel electrophoresis of purified His-tagged gp2.5-WT and gp2.5-NT confirmed that both gp2.5-NT and gp2.5-WT were selectively retained on the column and successfully eluted when high concentration imidazole was applied (Figure 6, lanes 4 - 9). In contrast, the flow through (FT) and wash (W) fractions did not contain significant amounts, if any, of the protein of interest, demonstrating that the entire amount of His-tagged protein was purified.

Purity of the dialyzed and pooled fractions was confirmed by gel electrophoresis. All lanes loaded with either gp2.5-WT or gp2.5-NT showed a single distinct band aligning with the appropriate MW marker for gp2.5 proteins, indicating all dialyzed gp2.5 proteins were efficiently purified. No additional protein bands were observed, indicating that the proteins were efficiently purified.

Figure 6. Quality assessment of the purified proteins.



A - Purity assessment of protein preparations. **1** - gp2.5-WT, **2** – gp2.5–NT, **3** - protein ladder.
B – ssDNA –binding assay. **1** - free DNA (--), **2** - ssDNA shift in presence of gp2.5-WT (*), **3** -ssDNA shift in presence of gp2.5-NT (**).

Pure gp2.5-WT and gp2.5-NT were determined to have maintained the function of binding ssDNA. Both complexes (labeled with "*" and "**" in Figure 6B) moved through the agarose gel more slowly than free DNA (--) thus demonstrating that the isolated proteins are able to bind ssDNA. Consistent with literature, gp2.5-NT caused a bigger shift in DNA mobility, which reflects its ability to bind DNA with higher affinity compared to the wild type.

Gel electrophoresis confirmed that the desired digestion products of pET19b PPS and pET17b, coding for gp2.5-NC was successfully produced (Figure 3). Currently, the selection process for the clone containing the correct and pET19.b PPS-gp2.5-NC plasmid is still in progress. Once obtained the DNA binding abilities of all three versions of gp2.5 will be evaluated.

The results of these studies will further the understanding of the molecular interactions of ssDNA binding proteins as well as other proteins with flexible charge tails which holds promise to impact the fields of bacterial resistance and cancer biology.

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